Isolation and Identification of Coliform Bacteria from Spicy Yogurt Milk and Plain Butter Milk sold in Different markets of Dhaka City

B.S. THESIS

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Declaration

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled "Isolation and Identification of Coliform Bacteria from Spicy Yogurt Milk and Plain Butter Milk sold in Different markets of Dhaka City" submitted by the undersigned has been carried out under the supervision of Zubaida Marufee Islam, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and any part of this thesis has not been submitted to any other institution for any degree or diploma.

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Ariful Azam

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Abstract

Spicy yogurt milk and plain butter milk are two of the most popular drinks in the south Asian region, especially in Bangladesh and India. These two drinks are widely consumed by people of this region as beverages in all seasons as it is a good source of nutrients in an affordable price. In Bangladesh, these drinks are so connected with our culture that in occasion like weddings these two drinks are served. For healthy diet plans, these two drinks can be a good option. However, because of hygiene and making procedure these drinks can become so unhealthy that it can be harmful for human health. The study was conducted to find out the quality of spicy yogurt drink and plain butter milk sold in the market of Dhaka city. In this study, total 31 (16 spicy yogurt milk and 15 plain butter milk) samples were collected from different markets and food shops of Dhaka city to check their microbial quality. The microbial quality was evaluated by quantifying total viable count, total coliform and fecal coliform count. During the study, it was found that out of all the 31 different samples of spicy yogurt drink and plain butter milk 1 spicy yogurt milk and 2 plain butter milk showed presence of *Pseudomonas spp. Escherichia coli* was found in other 2 plain butter milk and spicy yogurt milk samples. Another 2 spicy yogurt milk samples showed the presence of *Staphylococcus spp. Klebsiella spp.* was found in 2 spicy yogurt milk and 1 plain butter milk samples. One of the samples of spicy yogurt milk showed the presence of *Staphylococcus aureus* which is skin disease causing bacteria and can be found on affected person's skin. Some of the other organisms such as *Salmonella spp.* and *Listeria spp.* was found in 1 sample and *Shigella spp.* was found in other 3 samples. After the identification antibiotic susceptibility test was done to find out if any resistance strain is present. During the susceptibility test it the presence of multi-drag resistance microorganisms such as *E*scherichia-*coli and Pseudomonas spp.* were confirmed. Although from this study it can be said that, the microbial quality of the samples were not good for human health because of presence of different disease causing microbes at a very high level. The main purpose of this study was to create awareness among the consumers about the quality of the drink that they are taking and towards the seller who are providing low quality products to the society. The government should take some preventive steps and introduce some strict rules towards the manufactures and sellers so that they maintain proper hygiene. It is very important to maintain proper hygienic conditions for personal health benefit.

Keywords: Spicy yogurt milk, plain utter milk, coliform, fecal coliform, multi-drag resistance.

Chapter 1

Introduction

1.1 Introduction

Milk and dairy foods are highly nutritious foods. They contain a unique package of nutrients that are an essential part of a healthy eating plan. It is the one food for which there seems to be no adequate substitute and is one of the widely consumed products. Milk products such yogurt, spicy yogurt milk, butter milk are also rich in proteins, spices and minerals which made them widely expectable for their nutrient values. Milk is an ideal medium for microorganism growth and they can make it inconsumable for humans. Milk and, as we as milk products are consider as ideal food for human, especially for children (Varro *et at,* 1981). Milk and other milk products is highly susceptible to contamination by microorganisms and it is also a suitable medium for the rapid growth and multiplication of bacteria at favorable temperatures (Meghaand S.V. and Annadurai B., 2014). That's why it is important to know what particular microorganisms can potentially contaminate milk products because in South-Asian countries, improper processing conditions of dairy products are very prevalent making the products unhygienic. According to Us public health milk and milk products should not contain more than 20,000 bacteria ml^{-1} and 10 coliform bacteria ml^{-1} (Bhowmick *et al,* 2006).

Milk and milk products such as cheese, yogurt butter, butter milk, spicy yogurt milk, and raw milk cream can contain different types of microorganisms as well as pathogenic bacteria like *Pseudomonas spp., Staphylococcus spp., Escherichia coli, Shigella spp., Klebsiella spp.* and many more. Pathogens can be present in freshly drawn milk and it can spread further during handling. (Marth, 1969). Even after proper sanitation and handling measures taken under proper regulatory control some milk products poisoning has been reported (Credit *et al,* 1972). Different species of *Salmonella spp.* cause a variety of diseases by consuming milk and milk products (Bello, *et al,* 1976). There are few reports available about contamination of *Escherichia coli* and *P. paucimobilis* in fermented milk and yogurt (Hekmat and Macmohoan, 1997). However because of their metabolic versatility and common way of recovery from milk based products (Wessels et al., 1989; Shelly *et al,* 1986; Postupa and Aldova, 1984) they can survive in milk products of low pH (Cangella *et al,* 1999). The people of Bangladesh frequently use a variety of milk products such as cheese, whey and spicy yogurt milk. Spicy yogurt milk is a common milk product in Bangladesh which contains some spicy ingredients like master oil,

Piper nigrum paste curd, capsicum powder, *Coriandrum sativum* powder and different taste salt. That's why the present study was taken place to check microbial quality and antibiotic sensitivity of the microorganisms.

1.2 Milk

Milk is one of the highly consumed food products around the world. In 2011 dairy farms around the world produced about 730 million tons of milk. Milk is the very first food served on the earth and it is the most satisfactory food substances gifted by nature. It is the food which has no substitute. It is a complex mixture of carbohydrates, proteins, lipidsand other organic compounds and inorganic salts. Proteins like alpha-s-casein, beta-casein and kappa-asein, also alpha lactalbumin and beta lactoglobulin can be found in milk (Muean Aslam and Walter Hurley, 1996). Because of the consumption rate around the world it is called the "Liquid Dimond"

1.3 Butter milk

Butter milk is the liquid left over when making butter from milk. This sour tasting drink has been shown to have some health benefits. This contains lipids, proteins and vitamins which are water soluble (Hunziker O.F., 1923). A recent study showed buttermilk can reduce the growth of colon cancer cells. In addition to that, another study published by the journal Nutrition found that drinking buttermilk for a short period of times can reduce blood pressure in some individuals (Bradford, 2016). Also, as mentioned above, buttermilk also contains probiotic bacteria. This type of bacteria has been found to have many health benefits. Through the world various types of butter milk preparation methods are available. South Asian countries like India and Bangladesh it is also known as traditional butter milk. Diseases associated with digestion and digestive problems butter milk is very useful (Nirgude R., 2013). Butter milk is very rich source of potassium, calcium, phosphorus and vitaminB12 which are very good medicinal elements for digestion problems (Wagle *et al*, 2013).

1.4 Spicy Yogurt Milk

Spicy yogurt milk is one of the most consumed traditional drinks in South-Asian countries, such as Bangladesh, India, Sri Lanka and many more. Starting from a small reunion to big festivals like weddings, this beverage is an important beverage item. This is a common milk product in Bangladesh which contain some spicy ingredients like master oil, *Piper nigrum* paste curd, capsicum powder, *Coriandrum sativum* powder and different taste salt. It has a wide range of health benefits because it helps in digestion. It also contains probiotic bacteria. Also, the ingredients of spicy yogurt milk such as pepper, mint are widely known for their anti-microbial properties.

1.5 Prevention of Spoilage in Milk

In the beginning of advancement of the business dairy industry, milk was delivered under significantly less sterile conditions than are utilized today. Improvements among the main half of the twentieth century made huge diminishments in the rate of deterioration of crude milk and cream, by making it feasible to collect milk from farmers and shipments of crude milk over long separations with negligible growth of bacteria (William H.S., 2010). Spoilage ability of *Pseudomonas* spp. can be affected by rapid cooling and quick use of it is widely accepted (Jaspe *et al*, 1995).

Addition of carbon dioxide into milk and milk products can reduce the growth rate of many bacteria (Dixon & Kell, 1989). To keep the optimum quality of raw milk (King and Mabbitt, 1982) added CO2 and got a satisfactory result. In another study by (Loss and Hotchkiss, 2002) found that growth rates of both *Pseudomonas fluorescens* and the spores of *B. cereus* was so low during heating of the raw milk.

There are other alternative ways for the pasteurization of milk, like microwave heating, UV radiation, ohmic heating, pulsed electric fields, electron beam irradiation, infrared processing

and high voltage arc discharge. This ways of treatment can be use combining with other treatments. However, all the processes need to be verified with combine technique to achieve the critical processing limits and challenges (NACMCF, 2006).

1.6 Diseases caused by the microbes

Milk, as well as other milk products such as butter milk, yogurt milk, and butter can contain a large number of microbes with greater diversity and serves as a great growth medium (P. L. Ruegg, 2003). Bacteria can be the causative agent of microbial spoilage of milk products. Because of low pH of most of the milk products, bacteria such as *Shigella* spp., *Pseudomonas spp., Salmonella spp.,* can grow easily in them.

Pseudomonas spp. is gram negative bacteria. Morphologically they are vibrios and enteric bacilli. They are motile bacterial species because they have peritrichous flagella. *Pseudomonas spp.* is aerobes in nature and can be found widely in water, soil, skin and almost all the manmade environments. *Pseudomonas aeruginosa* can cause cystic fibrosis, urinary tract infection and also other external and internal of human body. *Pseudomonas spp.* is also opportunistic bacteria. Not every time they cause infection. They can remain silent inside host body but whenever they get the chance they can create worst situation. *P. aeruginosa* is one of its strains which is well known for its unique and advance multi-drag resistance mechanisms. This strain is associated with serious illness especially hospital acquired infection. *Pseudomonas spp.* is predominate in raw milk and play a major part in milk spoilage (Muir *et al,* 1979; Griffiths *et al,* 1987).

Escherichia coli is a gram negative bacterium which can be found in lower intestine of warm-blooded animals. A large variety of *E. coli* is present but most them are not infectious. They are harmless. *E. coli* are also opportunistic microbes. Some types of E. coli such as E. coli 0157:H7 can create intestinal infection. Some other strains can cause bloody diarrhea, severe kidney failure which can cause death. Humans and animals can be infected by eating foods or drinking water which is already contaminated with *E. coli.*

Staphylococcus aureus are gram positive, non-motile, small round shaped cocci. They can be frequently found in respiratory tract, nose and skin. They can grow without the presence of oxygen because they are facultative anaerobes. Pathogenic strains can cause infection by producing a cell surface toxin protein that binds with the antibodies and deactivates them. *Staphylococcus aureus* can spread by skin-to-skin contact, objects such as towels, cloths and other equipment that are used by the infected person. They can lay dormant in host body for several years undetected. *Staphylococcal* scalded skin syndrome is a severe skin infection which can be seen in new born babies. On the other hand antibiotic resistant strains such as methicillin-resistant *S. aureus* (MRSA) is now became a headache for worldwide clinical aspects. (Akbar and Anal, 2013).

Salmonella spp. are gram negative, rod-shaped (bacillus) bacterium. *Salmonella enterica* can cause four diverse clinical signs: bacteremia, gastroenteritis, asymptomatic carrier state and enteric fever. It is more common in youngsters less than 5 years old. Gastroenteritis is also known as food poisoning showing the symptoms such as vomiting, sudden nausea, abdominal cramps, headache, diarrhea and high fever. In Asia non-typhoid salmonellosis is more common especially in industrialized countries. Their primary hosts are animals such as cattle, wild birds, flies as well as pets. The final hosts are human. Humans are the only known hosts for *Salmonella Typhi.* Humans only get infected when they consume contaminated water and foods such as meat, milk, egg products, milk products which are contaminated with infected faeces as well as infected animals. Certain host carries the bacteria for years.

Klebsiella is a gram negative, rod-shaped, non-motile bacterium. This species can be found everywhere in nature. *Klebsiella* is an opportunistic bacterium and primarily they attach those people who are immunocompromised and suffering from different disease such as diabetes. They can be found in surface water, plants and soil, as well as in mucus of humans. *Klebsiella pneumonia is the most known pathogen among all the other species which cause human disease. Klebsiella spp.* also can cause septicemia, intensive care unit infection, and urinary tract infection.

There are other types of pathogenic bacteria present in the environment and they can cause various diseases to humans. All the pathogens mentioned above and the diseases caused by them can be treated with different types of antibiotics but some of them can show multi-drug resistance which is a very big problem. Vaccines are still unavailable for some of the pathogenic strains.

1.7 Literature review:

Malek *et al*, (2015) conducted their study to examine the likelihood of microbial contamination within some common milk products consumed by the locality of the city of Dhaka, Bangladesh. A total of 10 milk and milk products were collected aseptically from different shops within the city of Dhaka. Their samples included matta, sweetened yogurt, lassi and many other dairy products. All samples exhibited the presence of bacterial and fungal contamination within a range of 10^2-10^4 cfu/ml and 10^2-10^3 cfu/ml, respectively. Among specific pathogens, *Staphylococcus spp*. was noticed to be the predominant ones and was recovered from 9 samples out of 20 samples in a range of 10^2-10^3 cfu/ml. *Klebsiella spp*. and *Vibrio spp.* were found within 6 and 9 samples, respectively. An antibiogram test of eleven common antibiotics was also conducted for the pathogenic isolates. The antibiotic susceptibility test showed that all the pathogenic bacteria were resistant against the antibiotics of which several isolates showed multi-drug resistant (MDR) trait.

Bhowmick *et al*, (2006) also studied cheese, borhanii and whey samples of Dhaka Metropolitan City for microbial determination. The samples revealed the presence of coliform bacteria. However, coliform bacteria seemed to be most prevalent in cheese and borhanii than in whey samples. An antibiogram test was also carried out with five different antibiotics against the bacterial isolates. All the isolates were resistant to streptomycin and sensitive to gentamycin. The majority of the isolates were sensitive to chloramphenicol and tetracycline but four isolates were resistant to ampicillin.

Afroz *et al*, (2013) investigated twelve powder milk samples from different areas of Dhaka city and carried out antibiotic susceptibility pattern of isolated *Escherichia coli* and *Staphylococcus aureus* for twelve antibiotics. Their study revealed that *E. coli* was found in 11 samples and *Staphylococcus aureus* was isolated from 6 samples. Isolated *E. coli* were resistant to 5 antibiotics and *Staphylococcus aureus* isolates were resistant to six antibiotics.

Ahmed *et al*, (2015) carried out an investigation on quality evaluation of stirred yoghurt flavoured with Guddaim fruit. The ingredients and fruit puree were collected from different places of Khartoum, Sudan. Yogurt manufactured with Guddaim fruit was produced in their university laboratory. In their research, all the yoghurt samples were stored at 4 ºC for 10 days and chemical, microbiological and sensory characteristics were carried out at 10-day intervals. Dilution was done maintaining a ratio of 1:10 (v/v) and used for total viable bacteria (TVB), coliform bacteria, *Staphylococcus aureus* and yeasts and molds counts. For coliform bacterial count MacConkey agar medium was used and plates were incubated at 32ºC for 48 hr (Christen *et al*, 1992). Also, Mannitol salt agar medium was used for *Staphylococcus aureus* count and the plates were incubated at 32ºC for 48 hr (Flowers *et al*, 1992). During their study coliform bacteria count ranged between Log 3.15 cfu/gm in the control and Log 3.69 cfu/gm in Treatment 1 (5% v/v Guddaim fruit extract), and the count showed a decrease in day 7 (Log 3.06 cfu/gm) before they increasing to the end. On the other hand, *S. aureus* count was found to be Log 3.70 cfu/gm in Treatment 3 (10% v/v Guddaim fruit extract) and Log 4.44 in the control. The count was increased during storage from Log 4.07 cfu/gm at day 1 to Log 4.16 cfu/gm at day 10 with a slight decrease in day 7 (Log 3.90 cfu/gm). It was suggested that the environmental contamination and heat treatment during preparation of yoghurt might be the reason for the presence of these microorganisms.

Ihemeje *et al*, 2015 conducted their study on production and quality assessment on flavored and spiced yogurts. The samples of yogurt were produced according to International Standard of yogurt in their university laboratory, Owerri, Nigeria. They carried out mineral, microbiological, organoleptic and statistical analyses. In their microbiological analysis of samples, they determined the microbial load (coliform, bacteria and fungi load). The total bacterial count was lesser in pepper fruit spiced yogurt and ginger spiced yogurt than other fruit flavored yogurts.

And the result was the same for the coliform count as well. However, in their study, it was mentioned that a maximum count of 10^2 cfu/ml of coliform group bacteria was allowed in yogurt. So, samples with value less than 10^2 cfu/ml were considered to be safe for consumption. But absence of coliform could help extend the shelf-life of the dairy products.

Das *et al*, 2015 evaluated the microbial load and the quality of the eighty-seven dairy samples collected from different locations of Dhaka city. The study considered the total viable count, total coliform count and total fungus count of the different dairy samples. The coliform count of the yogurt was 9.5×10^3 cfu/g which was undesirable according to the FDA standard. On the other hand, the mean viable count of the borhani was significantly higher than raw and UHT milk.

1.8 Aim of the study

The aim of this research was to evaluate and check the microbial quality and quantity of the samples of spicy yogurt milk and plain butter milk. Considering the poor hygiene of the local food markets, this study was directed to emphasize the need for the establishment of an efficient quality control for the safe guard of human health as well as careful selection of antibiotics for drug resistant strains.
Therefore, the main objectives of this study were:

- Isolation of pathogenic bacteria from samples of spicy yogurt milk and plain butter milk
- Identification of the bacterial isolates using different biochemical tests
- Testing of the isolates for antibiotic sensitivity pattern to identify multi-drug resistant microorganisms.

Chapter 2

Materials and Methods

2.1 Study place

All the laboratory works regarding this research was done in the microbiology and biotechnology research laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

2.2 Study period

The research work was carried out from October, 2016 to June, 2017.

2.3 Materials

2.3.1 Media

Different types of media were used for selective growth, enrichment culture, and identification of specific properties and characteristics of different microorganisms. All the media preparation and sterilization were done according to standard recipe and protocol.

2.3.2 Biochemical test media

Different specific biochemical media were prepared for different biochemical tests. About 33 different antibiotic discs were used for identifying antibiotic sensitive and resistant bacteria.

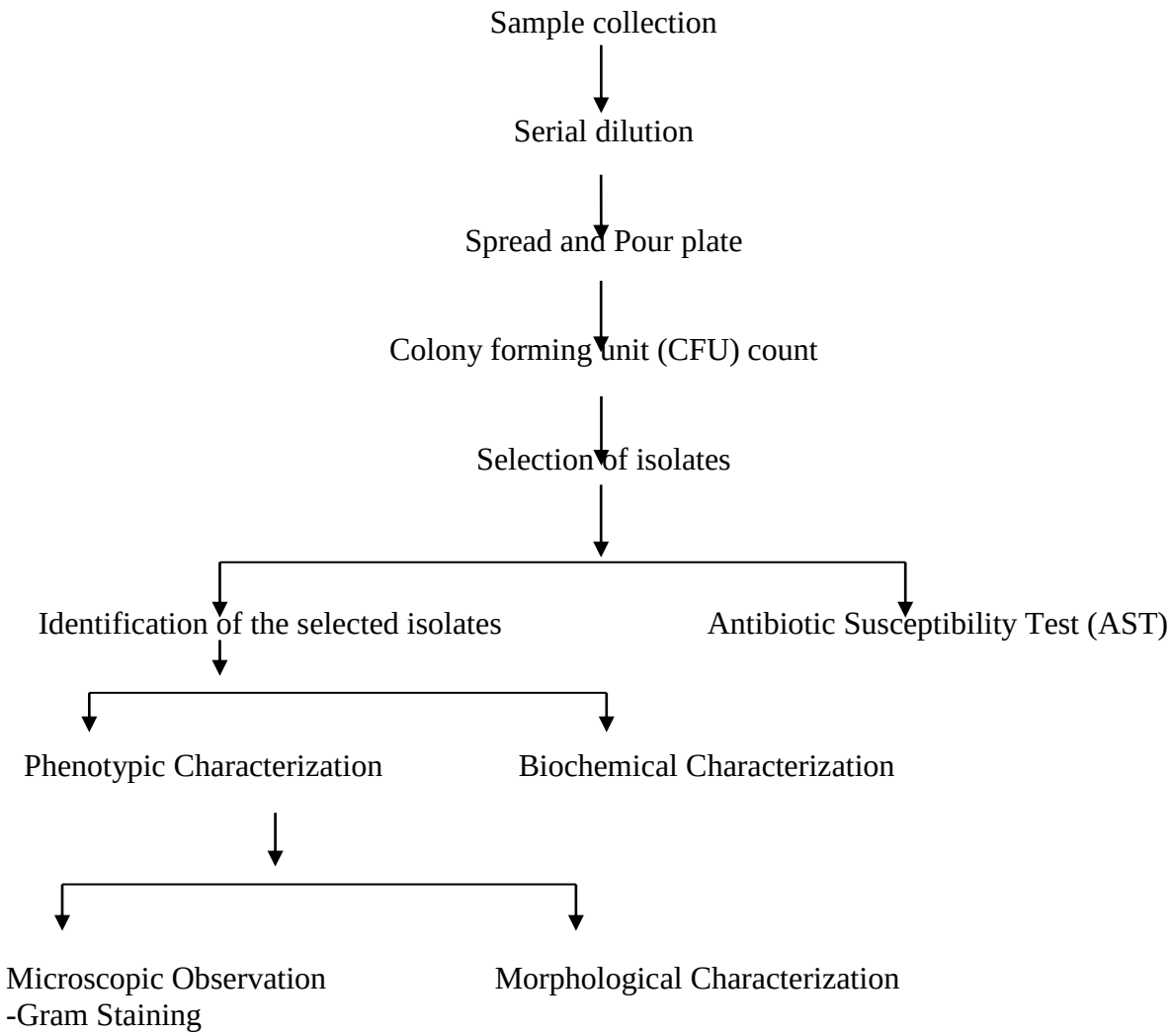
2.3.3 Antibiotic disc

Table 1: List of antibiotics and their zone ranges

Serial no.	Antimicrobial agent	Disk code	Disk potency (µg)	Range		
				Resistance (mm)	Intermediate (mm)	Susceptible (mm)
1.	Amikacin	AK	30	14	15-16	17
2.	Amoxicillin	AML	10	13/19	14-17	20
3.	Ampicillin	AMP	10	13/28	14-16	17/29
4.	Azithromycin	AZM	15	13	14-17	18
5.	Aztreonam	ATM	30	15	16-21	22

6.	Cefepime	CPM	30	14	15-17	18
7.	Cefoxitin	FOX	30	14	15-17	18
8.	Ceftazidime	CAZ	30	14	15-17	18
9.	Ceftriaxone	CRO	30	13	14-20	21
10.	Cephalexin	CL	30	14	14-18	19
11.	Chloramphenicol	C	30	12	13-17	18
12.	Ciprofloxacin	CIP	5	15	16-20	21
13.	Clindamycin	DA	2	14	15-20	21
14.	Cloxacillin	OB	5	15	16-19	20
15.	Co-trimethozole/sulfamethoprim	COT	25	10	11-15	16
16.	Doxycycline	DO	30	15	16-19	20
17.	Erythromycin	E	15	13	14-22	23
18.	Gentamicin	CN	10	12	13-14	15
19.	Imipenem	IPM	10	13	14-15	16
20.	Levofloxacin	LEV	5	13	14-16	17
21.	Minocycline	MH	30	14	15-18	19
22.	Nalidixic acid	NA	30	13	14-18	19
23.	Netilmicin	NET	30	15	16-19	20
24.	Nitrofurantoin	NIT	300	14	15-16	17
25.	Norfloxacin	NOR	10	12	13-16	17
26.	Oxacillin	OX	1	10	11-12	13
27.	Penicillin-G	P	10	14/28	12/21-21/28	15/19
28.	Piperacillin-tazobactam	TPZ	110	17	18-20	21
29.	Streptomycin	S	10	11	12-14	15
30.	Tetracycline	TE	30	14	15-18	19
31.	Tobramycin	TOB	10	12	13-14	15
32.	Trimethoprim/ Sulfamethozole	SXT	25	13	14-18	19
33.	Vancomycin	VA	30	14	15-16	17

2.4 Flowchart of the study design



2.5 Methods

2.5.1 Sample collection

Spicy yogurt milk and plain butter milk samples were collected from different locations of Dhaka city such as Motijhil, Mohakhali, Gulshan, Mirpur, Dhanmondi and Old Dhaka. For this research work total forty-six samples were collected and out of them twenty one samples are spicy yogurt milk and other fifteen samples were plain butter milk and all of them are collected from different food markets. All the samples were collected in sterilized Duran bottle to avoid contamination.

2.5.2 Sample processing

After collecting the samples, each of their pH was measured. The samples kept in 37^0 incubator for 24 hours in the Duran bottles to obtain better results. It helped to reproduce of the microorganisms in numbers. After the 24 hours incubation period serial dilutions were done from the samples and spread plate, pour plate method was done to see the growth of different microorganisms.

2.5.3 pH measurement

At first three beakers were rinsed with water and 70% ethanol, dried and labeled (sample, control and distilled water). After rinsing the pH meter with distilled water it was dipped into the beaker poured with juice sample. After 30 sec the reading of the pH meter was noted down and the pH meter was rinsed with distilled water and ethanol, dried and switched off. The process was followed for next all samples. The table below contains examples of substances with different pH values. (Decelles, 2002; Environment Canada, 2002; EPA, date unknown)

Table 2: The pH Scale; Some Examples of substances with different pH values

pH Value	H+ Concentration Relative to Pure Water	Example
0	10 000 000	battery acid
1	1 000 000	gastric acid
2	100 000	lemon juice, vinegar
3	10 000	orange juice, soda
4	1 000	tomato juice, acid rain
5	100	black coffee, bananas
6	10	urine, milk
7	1	pure water
8	0.1	sea water, eggs
9	0.01	baking soda
10	0.001	Great Salt Lake, milk of magnesia
11	0.000 1	ammonia solution
12	0.000 01	soapy water
13	0.000 001	bleach, oven cleaner
14	0.000 000 1	liquid drain cleaner

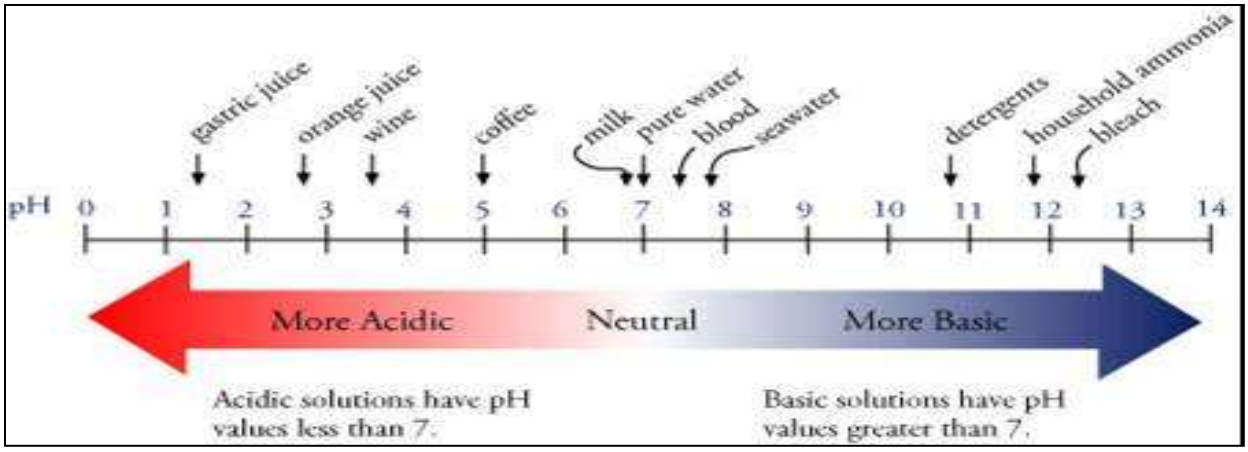


Figure 1: pH scale.

2.5.4 Serial dilution

Test tubes containing 9 ml of physiological (0.9% NaCl) saline water were autoclaved before use. Tenfold serial dilution of the spicy yogurt milk and plain butter milk samples were prepared in autoclaved saline water. Initially, 1 ml of spicy yogurt milk was mixed with 9 ml of saline water in a test tube in order to dilution 10^{-1} and mixed with 9 ml of saline in it by repeated pipetting in order to make tenfold dilution. Again, 1 ml from the 10^{-1} test tube was transferred to 10^{-2} labeled test tube and mixed with 9 ml saline solution in it by repeated pipetting. This action was repeated for the test tubes labeled as 10^{-3}, and 10^{-4}. For plain butter milk dilution, exactly same procedure was followed.

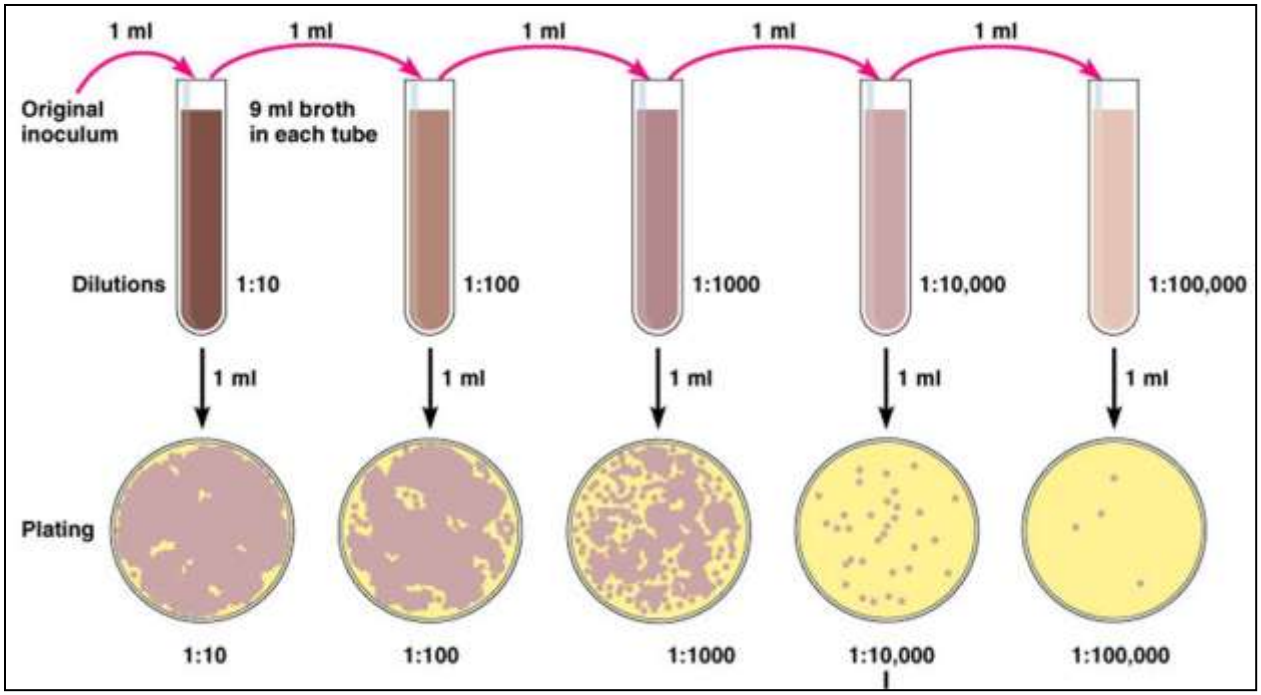


Figure 2: Serial dilution.

2.5.5 Spread plate method

After finishing serial dilution, five Nutrient agar plates were labeled as raw, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, three Mannitol Salt agar plates, three MacConkey agar plates and three XLD agar plates were labeled as raw, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. From each of the diluted sample test tubes 0.2 ml of sample from the test tubes labeled raw, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were added on the respective plates and the drops were spread using spread plate technique with a spreader. All plates were then incubated at 37◦C for 24-48 hours. After the incubation period the plates showing colonies were counted and noted down.

2.5.6 Pour plate

The test tubes labeled 10^{-1}, 10^{-2} and the raw sample, from each of the test tubes 2 ml inoculums were added on the respective MFc agar plates. Using pour plate method MFc agar plates were prepared. In the pour plate method, an inoculum was to be added to melted and cooled agar. The

agar inoculum mixture was then poured into a sterile petri dish. In the agar mixture all the isolated cells were to be trapped when it would solidify. These isolated cells would give rise to isolated pure colonies of microorganisms. A colony would be a visible mass of microorganisms growing on a solid medium.

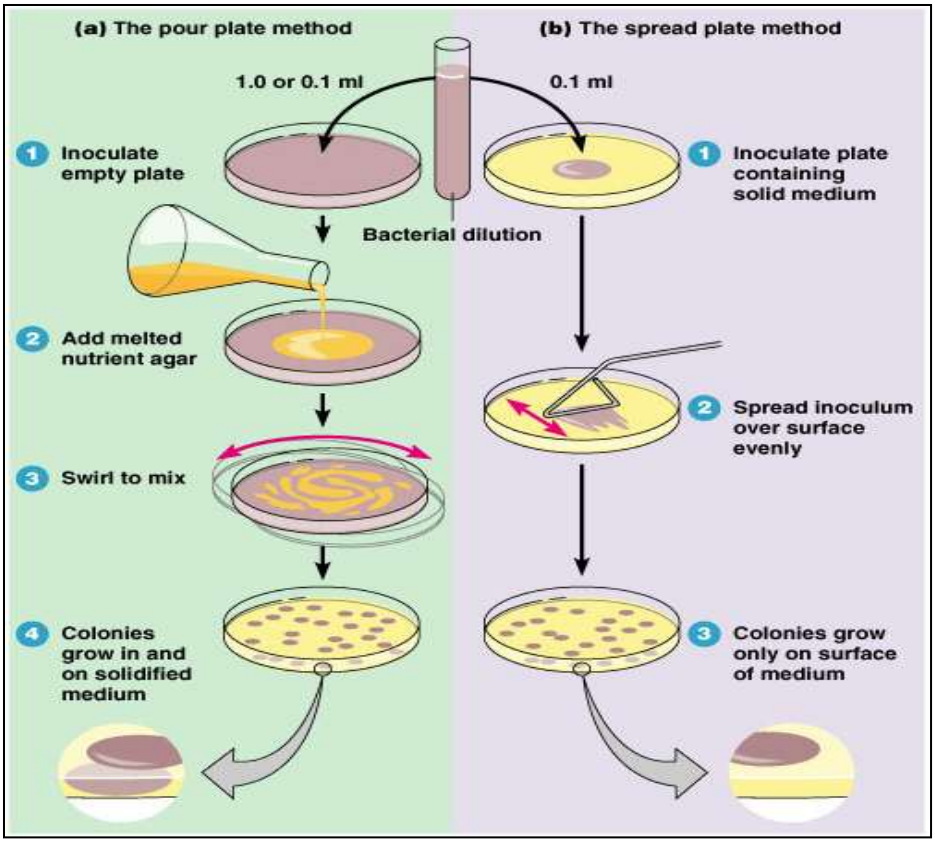


Figure 3: Pour plate and spread plate method.

2.5.6 Morphological characterizations of bacteria

Nutrient agar was prepared and autoclaved at 121◦C, 15 psi. The media was then dispensed into sterile petridis while liquid and left for a while get solidify. Using sterile technique, a NA agar plate was streaked by picking a loop full of colony of 24-hour fresh pure culture with an inoculating loop by means of three quadrant streak plate method to obtain isolated discrete colonies. The plates were then incubated at 37◦C for 24 hours. After the incubation period the

growth patterns of the bacteria were evaluated for size, pigmentation, form, margin, elevation and texture (Cappuccino and Sherman, 2005).

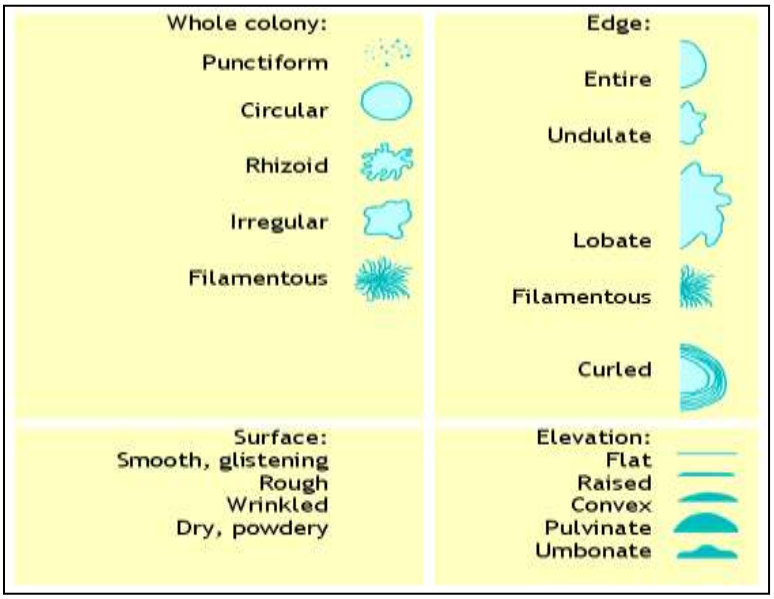


Figure 4: Colony morphology of bacteria.

2.5.7 Microscopic Observation of the bacteria

All the potential bacteria were observed under microscope in order to study their visual properties.

1. **Gram stain**

Gram staining was done to differentiate between two principle groups of bacteria:

- Gram positive
- Gram negative.

2. **Biochemical analysis**

2.5.8 Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology

laboratory manual (Cappuccino and Sherman, 2005). The biochemical tests performed were Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, Citrate utilization test), MIU test (Motility test, Indole test and Urease test), Nitrate reduction test, Catalase test, Oxidase test, Casein hydrolysis test, Gelatin hydrolysis test, Starch hydrolysis, Blood agar, Eosin methylene blue agar, and Cetrimide agar.

2.5.8.1 Triple Sugar Iron Agar test

Triple sugar iron test was done to differentiate among the different groups or genera of the Enterobacteriaceae based on the ability to reduce sulfur and ferment carbohydrates. Triple sugar iron slants were prepared in the test tubes by autoclaving at 15 psi 121◦C. Using sterile technique; small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab and streak inoculation method with an inoculating needle. The screw caps were not fully tightened and the tubes were incubated for 24 hours at 37◦C. (Cappuccino and Sherman, 2005)

2.5.8.2 Indole Production test

Indole production test was done to determine the ability of the bacteria to degrade the amino acid tryptophan by the enzyme tryptophanase. Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 48 hours at 37◦C. In order to test for indole production, 5 drops of Kovac's reagent was added directly into the tubes. (Cappuccino and Sherman, 2005)

2.5.8.3 Methyl red test

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products. MR-VP broth of 7 ml in each test tubes were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 hours at 37◦C. After 24 hours 3.5 ml from the culture tubes were transferred to clean test tubes for Voges- Proskauer test and the remaining broth were re-incubated for additional 24 hours. After 48-hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes to observe the immediate development of a red colour. (Cappuccino and Sherman, 2005)

2.5.8.4 Voges Proskauer test

Voges Proskauer test was done to differentiate further among enteric organisms such as E.coli, E. aerogenes, and K. pneumoniae by determining the capability of the organisms to produce non acidic or neutral end products such as acetylmethylcarbinol. To the aliquot of MR-VP broth after 24 hour incubation, 0.6 ml (12 drops) of 5% alpha naphthol (reagent A) was added followed by 0.2 ml (4 drops) of 40% KOH (reagent B). The tube was gently shaked to expose the medium to atmospheric oxygen (30seconds-1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. The test was read, but not beyond, one hour following the addition of the reagents. (Cappuccino and Sherman, 2005)

2.5.8.5 Citrate utilization test

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease. Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi 121◦C. Using

sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37◦C.(Cappuccino and Sherman, 2005)

2.5.8.6 MIU (Motility- Indole- Urease) test

MIU test was done to simultaneously determine the ability of the bacteria to produce indole, check motility and degrade urea by means of the enzyme urease. MIU media was prepared by autoclaving at 15 psi 121◦C. the media was cooled to about 50-55◦C and 100ml of urea glucose solution was added aseptically to 900 ml base medium. After that, 6ml solution was transferred to each sterile test tube and allowed to form a semi solid medium. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37◦C. (Cappuccino and Sherman, 2005)

2.5.8.7 Nitrate reduction test

Nitrate reduction test was done to determine the ability or inability of the bacteria to reduce nitrate (NO_3^-) to nitrite (NO_2^-) or beyond the nitrite stage using anaerobic respiration by the enzyme nitrate reductase. Nitrate broth of 6 ml in each test tubes were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 to 48 hours at 37◦C. After incubation, 5 drops of reagent A and 5 drops of reagent B was added to each broth. If there was no red colour development, a small amount of zinc was added to each broth. (Cappuccino and Sherman, 2005)
Note: Caution was maintained during the use of powdered zinc since it is hazardous.

2.5.8.8 Catalase test

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase. A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24-hour pure culture was placed onto the microscopic slide. 1 drop of 3% H_2O_2 was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation. (Cappuccino and Sherman, 2005)

2.5.8.9 Oxidase test

Oxidase test was done to determine the presence of the enzyme cytochrome oxidase in the bacteria. A small piece of filter paper was soaked in Gaby and Hadley oxidase test reagent and let dry. Using an inoculating loop, a well isolated colony from pure 24-hour culture was picked and rubbed onto filter paper and observed for colour change (Shields and Cathcart, 2010).

2.5.8.10 Gelatin hydrolysis test

Gelatin hydrolysis test was done to detect the ability of the bacteria to produce gelatinase. All the ingredients of the nutrient gelatin medium were mixed and gently heated to dissolve. Three milliliter from the media was dispensed in glass vials. The glass vials with the medium were then autoclaved at 121◦C, 15 psi. The tubed medium was allowed to cool in an upright position before use. Using sterile technique, a heavy inoculum of 24-hour old culture bacteria was stab inoculated into the tubes with an inoculating needle. The glass vials were then incubated at 37◦C and observed up to for 1 week. (Cappuccino and Sherman, 2005)

2.5.8.11 Starch hydrolysis test

Starch hydrolysis test was done to determine the ability of the bacteria to hydrolyze starch with the enzyme amylase. Starch agar was prepared and autoclaved at 121◦C, 15 psi. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a starch agar plate was streaked by picking a loopful colony of 24-hours old pure

culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 48 hours and the hydrolysis was observed using gram's iodine. (Cappuccino and Sherman, 2005)

2.5.8.12 Casein hydrolysis test

Casein hydrolysis test was done to determine the ability of the bacteria to produce the enzyme caseases and hydrolyze casein thereby. Distilled water and agar solution was taken in separate conical flasks and both were autoclaved at 121°C, 15 psi. Skim milk powder was then added to the autoclaved distilled water aseptically and boiled for 1 minute to dissolve completely. After that, the milk solution was mixed with agar solution. The media was added into sterile plates while liquid and left for a while to solidify. Using sterile technique, a milk agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37°C for 24 hours. (Cappuccino and Sherman, 2005)

2.5.8.13 Blood agar test

Blood agar test was done to determine the hemolytic capability of the bacteria by producing hemolysins and thereby lyse red blood cells. Blood agar base was prepared in a conical flask and autoclaved at 121°C, 15 psi. The nutrient agar medium was allowed to cool at 45-50°C and 5% (vol/ vol) sterile defibrinated sheep blood that had been warmed to room temperature was added and gently mixed avoiding air bubbles. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a blood agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37°C for 24 hours. After incubation, the plates were observed for gamma, beta and alpha hemolysis. (Cappuccino and Sherman, 2005)

2.5.8.14 Eosin methylene blue agar test

This test was done to select and isolate Gram negative organisms, and coliforms, and to differentiate among the family of Enterobacteriaceae. The main use of this test was to isolate fecal coliforms and to detect for fecal contamination. Using sterile technique, an EMB agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37°C for 24-48 hours. Slow growing species may require a day or two of additional growth. (Cappuccino and Sherman, 2005)

2.5.8.15 Cetrimide agar test

This test was used for determining the ability of an organism to produce fluorescein and pyocyanin (Antibiotica). Plates were labeled and marked according to the dish side bottom in what species would be in each section to observe the growth clearly. Using sterile technique, an Cetrimide agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37°C for 24-48 hours. Slow growing species may require a day or two of additional growth. (Cappuccino and Sherman, 2005)

2.5.8.16 Antibiotic resistance and susceptibility analysis

In clinical microbiology laboratory it is an important task to check the performance of antimicrobial susceptibility testing of significant bacterial isolates. The aim of this test is to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. Manual methods that provide flexibility and possible cost savings include the disk diffusion and gradient diffusion methods.

The disk diffusion susceptibility method is simple and practical and has been well-standardized. The test was performed by applying a bacterial inoculum of approximately $1–2\times10^8$ cfu/ mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentrations, paper antibiotic disks were placed on the inoculated agar surface.

Plates were incubated for 16–24 hours at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The results of the disk diffusion test were "qualitative," in that a category of susceptibility (i.e. susceptible, intermediate, or resistant) was derived from the test rather than an MIC.

The advantages of the disk method are the test simplicity that does not require any special equipment, the provision of categorical results easily interpreted by all clinicians, and flexibility in selection of disks for testing. It is the least costly of all susceptibility methods. The disadvantages of the disk test are the lack of mechanization or automation of the test. Although not all fastidious or slow growing bacteria can be accurately tested by this method, the disk test has been standardized for testing *Streptococci spp.*, *Haemophilus influenza* and *N. meningitidis* through use of specialized media, incubation conditions, and specific zone size interpretive criteria. (Wayne, 2009)

Chapter 3

Result

Spicy yogurt milk and plain butter milk are very common and widely popular in the south Asian region and it carries potential health benefits. On the other hand, just because of poor hygiene, quality and safety lots of question have been raised towards this two traditional beverage drinks. Some of the companies like Pran and Arong started producing spicy yogurt drink and plain butter milk for commercial purposes but most of the food shops are producing their own spicy yogurt drink and plain butter milk and they are not even concern about the quality of their products. On the other hand, majority people are more concern about the low price and nutritional benefits rather than the quality of the products. To conduct this study, total thirty-one samples (sixteen spicy yogurt milk and fifteen plain butter milk) were collected and examined for microbial analysis.

3.1 pH measurement of the samples:

Spicy yogurt milk and plain butter milk is highly rich in lactic acid. Because of the presence of the lactic acid the pH values of spicy yogurt milk was between 3.8 - 4.6 and for plain butter milk the pH value was between 4.2 - 4.6. The minimum pH values that allow the growth of lactic acid bacteria is pH 3.00 – 3.5, for acetic acid bacteria pH 3.00 – 4.5, and for enteric bacteria pH 3.00 - 4.00. Molds and yeasts grow in higher pH than this. Most of the spicy yogurt milk and plain butter milk samples have pH value between 3.5 – 4.5. This indicates that mostly acetic acid bacteria, lactic acid bacteria and enteric bacteria will grow easily in the samples.

3.2 Total bacterial count of the samples:

Microbial count of the samples is a very important part of the research because it gives an idea about the microbial load. After seeing the results, it is clear that maximum of the samples contained higher rate of microbial load.

Table 3: Total bacterial count of freshly collected spicy yogurt milk

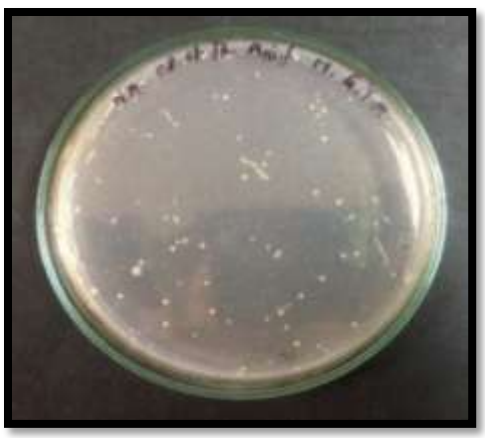
Sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Total Viable count(cfu/ ml)	2.3 x 10^3	4.5 x 10^6	6.2 x 10^5	3.2 x 10^5	1.6 x 10^5	2.0 x 10^6	1.8 x 10^2	5.1 x 10^4	4.4 x 10^6	3.0 x 10^6	1.1 x 10^6	5.5 x 10^5	4.1 x 10^5	2.1 x 10^3	5 x 10^4	6.8 x 10^5
Total Coliform Count(cfu/ml)	2 x 10^5	5 x 10^6	7.2 x 10^3	8.0 x 10^2	4.0 x 10^5	5.7 x 10^3	3.6 x 10^5	8.5 x 10^6	5.0 x 10^6	6.0 x 10^3	4.3 x 10^4	1.7 x 10^4	2.2 x 10^6	5.65 x 10^3	3.9 x 10^5	1.0 x 10^4
Fecal Coliform Count(cfu/ml)	Nil	2.8 x 10^3	Nil	Nil	3.7 x 10^3	5.0 x 10^3	1.1 x 10^4	Nil	Nil	Nil	6.2 x 10^3	1.1 x 10^3	1.7 x 10^5	Nil	Nil	Nil
Total Staphylococcal Count(cfu/ml)	2.1 x 10^2	3.05 x 10^3	1.7 x 10^5	6.6 x 10^5	4.8 x 10^4	1.4 x 10^5	1.2 x 10^5	2.5 x 10^2	3.55 x 10^2	1.75 x 10^2	2.3 x 10^3	1.4 x 10^4	4.4 x 10^2	1.65 x10^3	2.65 x 10^3	3.4 x 10^4
Total Salmonella count(cfu /ml)	Nil	Nil	Nil	Nil	2.6 x 10^4	Nil	Nil	Nil	3.8 x 10^4	Nil	1.0 x 10^4	Nil	Nil	1.2 x 10^4	Nil	Nil

The number 3 table showed the data of total viable count of spicy yogurt milk which is ranged from 1.1 x10^6 - 6.8 x10^5 cfu/ ml. Total coliform count ranged from 1.0 x 10^4 – 8.5 x 10^6 cfu/ ml. Total fecal coliform count ranged from 0 - 6.2 x10^3 cfu/ ml. As well as total staphylococcal count ranged from 1.7 x 10^2 – 6.6 x x10^5 cfu/ ml and total Salmonella count ranged from 0 - 3.8 x10^4 cfu/ ml

Table 4: Total bacterial count of freshly collected plain butter milk

Sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Total Viable count(cfu/ ml)	1.2 x 10^3	2.3 x 10^6	1.8x 10^4	2.9 x 10^5	1.1 x10^5	2.0 x 10^3	5.2 x10^2	2.1 x 10^3	6.2x 10^4	5.5x1$0^5$	1.0x 10^5	3.3x 10^5	2.2 x 10^5	4.3x1$0^3$	3.1x 10^4
Total Coliform Count(cfu/ml)	0.5 x 10^5	2.1x 10^6	2.7 x 10^3	Nil	Nil	7.5 x 10^3	2.6 x 10^5	5.9 x 10^6	Nil	Nil	2.7 x 10^4	1.7 x 10^4	2.2 x 10^6	5.65 x 10^3	Nil
Fecal Coliform Count(cfu/ml)	Nil	Nil	Nil	Nil	3.7 x10^2	5.0 x 10^2	1.1 x 10^4	Nil	Nil	Nil	Nil	1.1 x 10^3	1.7 x 10^2	5.2 x 10^3	Nil
Total Staphylococcal Count (TSC) (cfu/ ml)	1.6 x 10^2	Nil	2.5 x 10^3	6.2 x 10^1	4.1 x10^2	1.4 x 10^5	1.2 x 10^5	Nil	Nil	3.75 x 10^2	2.3 x 10^3	4.1 x 10^3	0.4 x 10^2	1.65 x 10^3	5.0 x 10^3
Total Salmonella count(cfu /ml)	Nil	Nil	Nil	2.6 x 10^2	Nil	Nil	Nil	Nil	1.7 x 10^3	Nil	Nil	Nil	Nil	Nil	Nil

The number 3 table showed the data of total viable count of spicy yogurt milk which is ranged from 1.1 x10^6 - 6.8 x10^5 cfu/ ml. Total coliform count ranged from 1.0 x 10^4 – 8.5 x 10^6 cfu/ ml. Total fecal coliform count ranged from 0 - 6.2 x10^3 cfu/ ml. As well as total staphylococcal count ranged from 1.7 x 10^2 – 6.6 x x10^5 cfu/ ml and total Salmonella count ranged from 0 - 3.8 x10^4 cfu/ ml.



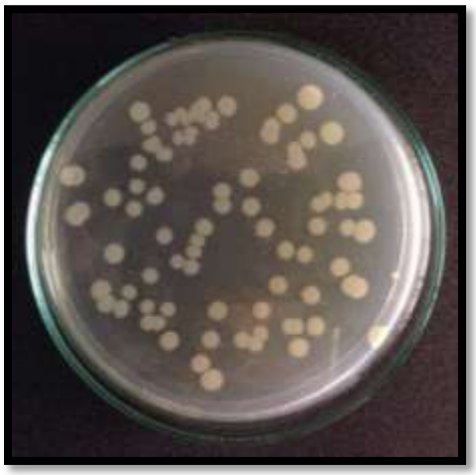
Spread plate and colony count of SYM sample 1

Spread plate and colony count of PBM sample 1

Spread plate and colony count of SYM sample 2

Spread plate and colony count of PBM sample 2

Spread plate and colony count of SYM sample 3



Spread plate and colony count of PBM sample 3

Figure 6: Spread plate and colony count of different sample.

3.3 Gram reaction and colony morphology

Gram reaction and colony morphology of different isolates collected from different samples were explained in this table. Colour of the colonies, their forms, margin reactions, elevations, gram reactions of those isolates and their shapes were analyzed are presented in the following table:

Table 5: Morphological characteristics of bacterial colonies and gram reaction

Bacterial isolates	Colour on Nutrient agar	Elevation	Margin reaction	Configuration	Gram reaction	Shape
SYM-S-7	White	Raised	Entire	Circular	Positive	Cocci
PBM-S-1	Green fluorescent	Flat	Filamentous	Rhizoid	Negative	Long single Rods
SYM-S-5	Off-white	Raised	Undulate	Irregular	Negative	Long rods
SYM-S-5	Bright yellow	Convex	Entire	Circular	Positive	Cocci
PBM-S-9	Bright yellow	Convex	Entire	Circular	Positive	Cocci
PBM-S-3	Green fluorescent	Flat	Filamentous	Rhizoid	Negative	Long single Rods
PBM-S-7	White	Flat	Entire	Circular	Negative	Short rods
SYM- S-3	Off-white	Raised	Undulate	Irregular	Negative	Long single rods
SYM-S-2	Off-white	Flat	Undulate	Irregular	Positive	Coccobacilli
PBM-S- 9	Off-white	Flat	Undulate	Irregular	Positive	Coccobacilli
SYM-S-11	Off-white	Convex	Entire	Circular	Negative	Cocci
SYM-S-7	Off-white	Flat	Entire	Circular	Negative	Coccobacilli
PBM-S-4	Off-white	Flat	Entire	Circular	Negative	Coccobacilli

SYM-S-16	Mucoid	Convex	Undulate	Irregular	Negative	Coccobacilli
SYM-S-3	Off-white	Raised	Undulate	Irregular	Negative	Long rods
SYM-S-4	Light yellow	Convex	Entire	Circular	Negative	Coccobacilli
SYM -S-6	Off-white	Convex	Entire	Circular	Negative	Cocci
SYM-S- 5	Bright yellow	Convex	Entire	Circular	Negative	Cocci in clusters
PBM-S-1	Mucoid	Convex	Undulate	Irregular	Negative	Coccobacilli
PBM-S-2	Green fluorescent	Flat	Filamentous	Rhizoid	Negative	Long single Rods
PBM-S-8	White	Flat	Entire	Circular	Negative	Short rods
SYM-S-14	White	Flat	Entire	Circular	Negative	Short rods
SYM-S-13	Off-white	Flat	Undulate	Irregular	Negative	Long rods in chains
PBM-S-15	Off-white	Raised	Undulate	Irregular	Negative	Long single rods

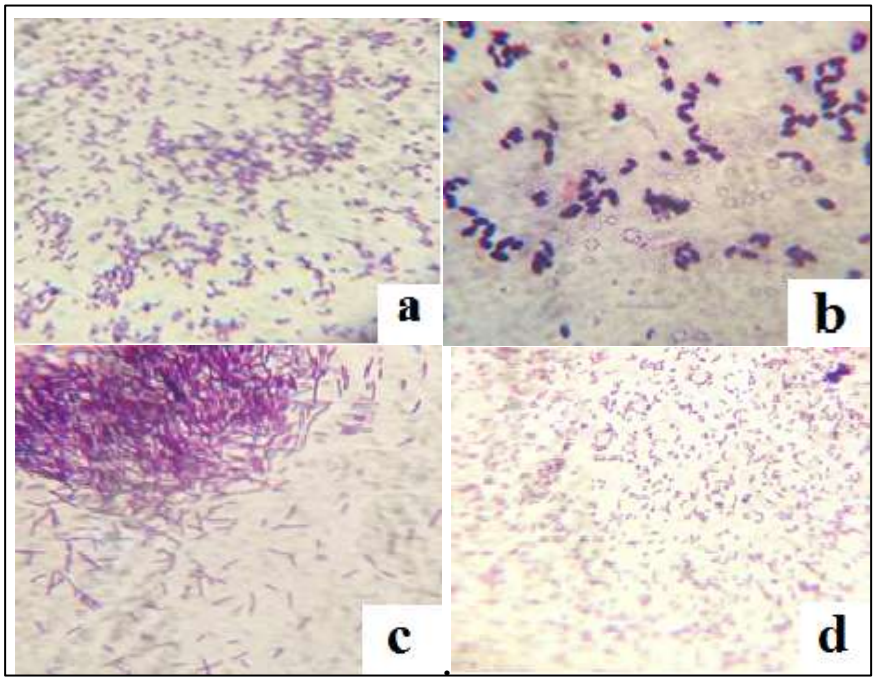


Figure 7: Gram reaction and shapes of different bacteria. **a.** Gram positive cocci. **b.** Gram positive cocci. **c.** Gram negative rod shape. **d.** Gram positive cocci.

3.4 Biochemical Characteristics of bacterial isolates of different samples

Suspected microorganisms were collected from the different samples. The selection of the microbes was done from different culture media according to their growth, morphology and visual form. Then the microorganisms were compared with the suspected one and selected one was then sub-cultured for biochemical tests. To obtain more specific and desired result different types of selective growth media were used to isolate specific microorganisms. All the biochemical tests were described with details in chapter 2 methods and materials section. The test results of the selected microorganisms are noted down with a chart and they are given below:

Table 6: Biochemical characteristics of bacterial isolates of different SYM and PBM																								
Isolates no.	**Isolates**	**Oxidase test**	**Catalase test**	**Indole**	**MIU**			**MRVP**		**Gelatin**	**Nitrate reduction**	**Simmon's citrate**	**Casein hydrolysis**	**Starch hydrolysis**	**Blood agar hemolysis**	**Eosin methylene blue**	**Cetrimide agar**	**TSI**						**Organism Interpretation**
					Motility	**Indole**	**Urease**	**Methyl Red**	**VogesProskauer**									**Slant/ Butt**	**Glucose**	**Lactose**	**Sucrose**	**H_2S production**	**Gas production**	
1	SYM-S-7	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	+	R/Y	+	-	-	-	-	*Staphylococcus aureus*
2	SYM-S-13	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-	R/R	-	-	-	-	-	*Bacillus spp.*
3	PBM-S-14	-	+	-	+	-	+	+	-	-	+	+	+	-	-	+	-	(B) R/Y	+	-	-	+	+	*Listeria spp.*
4	SYM-S-3	-	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	-	*Klebsiella spp.*
5	SYM-S-7	-	+	-	+	-	+	+	-	-	+	+	-	+	+	-	-	R/B	-	-	-	+	+	*Shigella spp.*
6	PBM-S-2	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	R/R	-	-	-	-	-	*Pseudomonas spp.*
7	SYM-S-2	+	+	-	+	+	-	+	-	-	+	+	+	-	-	+	+	B/B				+	+	*Salmonella spp.*

8	SYM-S-14	-	+	-	+	-	+	+	-	-	+	-	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli*
9	SYM-S- 16	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	R/R	-	-	-	-	-	*Pseudomonas spp.*
10	SYM-S-5	-	+	+	-	+	+	+	-	-	+	-	-	-	+	+	-	Y/Y	-	+	+	-	-	*Staphylococcus spp.*
11	SYM-S-16	-	+	-	-	+	+	+	-	-	+	+	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli*
12	SYM-S-4	-	+	+	+	+	-	+	-	-	-	+	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli*
13	SYM-S-5	-	-	-	+	-	+	-	+	-	+	-	-	-	+	-	-	R/R	-	-	-	-	-	*Bacillus spp.*
14	SYM-S-11	-	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	-	*Klebsiella spp.*
15	SYM-S-3	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-	-	R/R	-	-	-	-	-	*Bacillus spp.*
16	PBM-S-8	-	+	-	+	-	-	+	-	-	+	-	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli*
17	PBM-S-15	-	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	-	*Klebsiella spp.*
18	PBM-S-1	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli*

19	PBM-S-4	-	+	-	+	-	-	+	-	-	+	+	-	+	+	-	-	R/B	-	-	-	+	+	*Shigella spp.*
20	PBM-S-9	-	+	+	-	+	+	+	-	-	+	-	-	-	+	+	-	Y/Y	-	+	+	-	-	*Staphylococcus spp.*
21	PBM-S-3	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	R/R	-	-	-	-	-	*Pseudomonas spp.*
22	SYM-S-11	+	+	-	+	+	-	+	-	-	+	+	+	-	-	+	+	B/B				+	+	*Salmonella spp.*
23	SYM-S-6	-	+	-	+	-	+	+	-	-	+	+	+	-	-	+	-	(B) R/Y	+	-	-	+	+	*Listeria spp.*
24	SYM-S-5	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	R/R	-	-	-	-	-	*Bacillus spp.*
25	PBM-S-15	-	+	-	+	-	+	+	-	-	+	+	-	+	+	-	-	R/B	-	-	-	+	+	*Shigella spp.*
‘+’ = positive, ‘- ‘ = negative; ‘Glu’ = Glucose; ‘Lac’ = Lactose, ‘Suc’ = Sucrose, Y= Yellow, R= Red, B= Black, , SYM= Spicy Yogurt Milk, PBM= Plain Butter Milk, S= Sample																								

During the study after all the biochemical tests has been done it was found that *Staphylococcus aureus* was present in 1 spicy yogurt milk sample, *Escherichia-coli.* isolates was found in 4 different samples of spicy yogurt milk and plain butter milk, *Shigella spp.* and *seudomonas spp.* isolates was found in 3 different samples as well. *Bacillus spp.* was also found in 4 different samples. 1 *Salmonella spp*. was identified. Moreover, other microorganisms such as *Listeria spp.* have been identified.

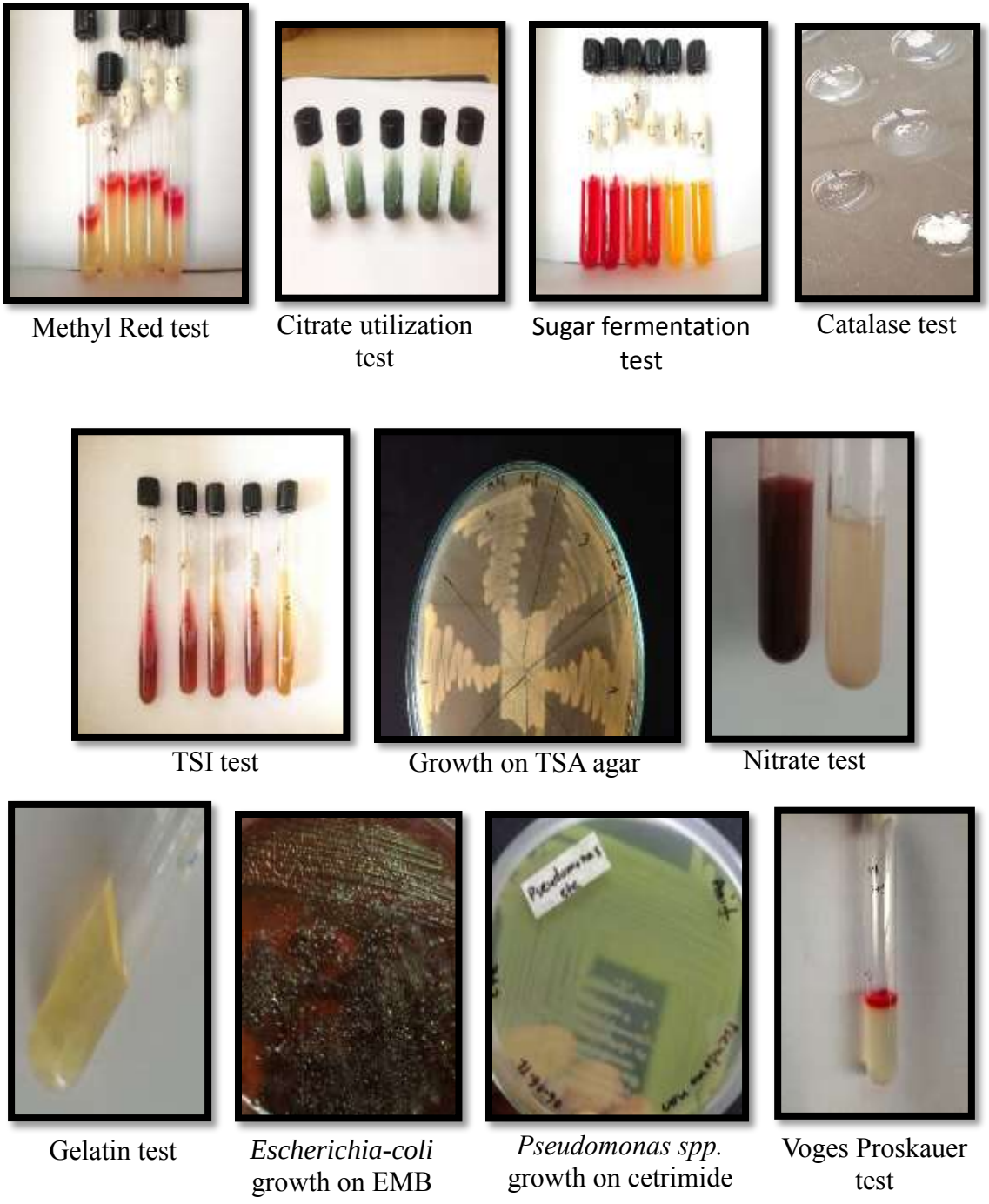


Figure 8: Different types of biochemical test results and microbial growth on selective media.

3.5 Antibiotic susceptibility test

All the isolates were selected for the antibiotic susceptibility test. Total twenty-one antibiotics were used to test the isolates sensitivity and resistance against them as shown in the table below. The table showing zone inhibition of different bacteria on the basis of their resistance zone range and sensitivity. Clear zone has been shown by some bacteria which means they are sensitive towards those antibiotics. On the other hand, some showed no clear zone at all. That means they are resistance towards those antibiotics. Some of them are called intermediate to some antibiotics. That means if the clear zone diameter is larger than resistance diameter scale and less than susceptible diameter then the specific bacteria is neither resistance nor susceptible to that particular antibiotic. The interpretation is given below:

Table 7: Antibiotic Susceptibility testing												
Antimicrobial agent	**Tested Organisms**											
	Staphylococcus aureus		***Pseudomonas spp.***		***Bacillus spp.***		***Shigella***		***E. coli***		***Salmonella spp.***	
	ZS	*INP*	*ZS*	*INP*	*ZS*	*INP*	*ZS*	*INP*	*ZS*	*INP*	*ZS*	*INP*
1. Amoxicillin	35	S	Nil	R	15	S	Nil	R	Nil	R	Nil	R
2. Cefoxitin	30	S	Nil	R	23	S	26	S	Nil	R	Nil	R
3. Ceftazidime	20	S	Nil	R	19	S	19	S	26	S	Nil	R
4. Ceftriaxone	27	S	Nil	R	15	S	39	S	Nil	R	31	S
5. Cephalexin	29	S	Nil	R	25	S	26	S	Nil	R	Nil	R
6. Chloramphenicol	26	S	Nil	R	26	S	13	S	28	S	28	S
7. Clindamycin	26	S	Nil	R	27	S	Nil	R	Nil	R	Nil	R
8. Doxycycline	34	S	Nil	R	24	S	Nil	R	14	S	30	S
9. Gentamicin	28	S	Nil	R	25	S	28	S	15	S	27	S
10. Imipenem	52	S	15	S	38	S	41	S	Nil	R	35	S
11. Kanamycin	29	S	Nil	R	23	S	27	S	Nil	R	30	S
12. Levofloxacin	35	S	Nil	R	28	S	22	S	29	S	39	S
13. Minocycline	34	S	Nil	R	19	S	14	S	18	S	23	S
14. Nalidixic acid	19	S	Nil	R	22	S	Nil	R	19	S	Nil	R
15. Netilmicin	34	S	Nil	R	23	S	30	S	13	S	29	S
16. Nitrofurantoin	26	S	Nil	R	18	S	15	S	Nil	R	Nil	R
17. Oxacillin	22	S	Nil	R	16	S	Nil	R	Nil	R	Nil	R
18. Penicillin-G	42	S	Nil	R	19	S	Nil	R	Nil	R	Nil	R
19. Rifampicin	34	S	Nil	R	16	S	14	S	Nil	R	12	S
20. Streptomycin	23	S	Nil	R	26	S	Nil	R	Nil	R	28	S
21. Tobramycin	22	S	Nil	R	20	S	23	S	17	S	30	S
ZS= Zone size, INP= Interpretation , S= Sensitive, I= Intermediate, R= Resistant, '-'= Not Done,												

An antibiogram test was carried out with twenty-one antibiotics. From the above table, it can be concluded that *Staphylococcus aureus* was the most sensitive among the six isolates followed by *Bacillus spp*. *Escherichia-coli* showed resistant to rifampicin, doxycycline, amoxicillin, streptomycin, clindamycin, cephalexin penicillin-G, oxacillin, kanamycin, cefoxitin, imipenem and nitrofurantoin. *Salmonella spp.* showed resistance to 9 different antibiotics. However, *Pseudomonas spp*. was surprisingly found to be the most resistant to antibiotics. *Pseudomonas spp.* showed resistance to twenty antibiotics and was only intermediate to imipenem. Imipenem, however, couldn't kill *Escherichia coli*. The largest zone of inhibition was shown by *Staphylococcus aureus* against imipenem.

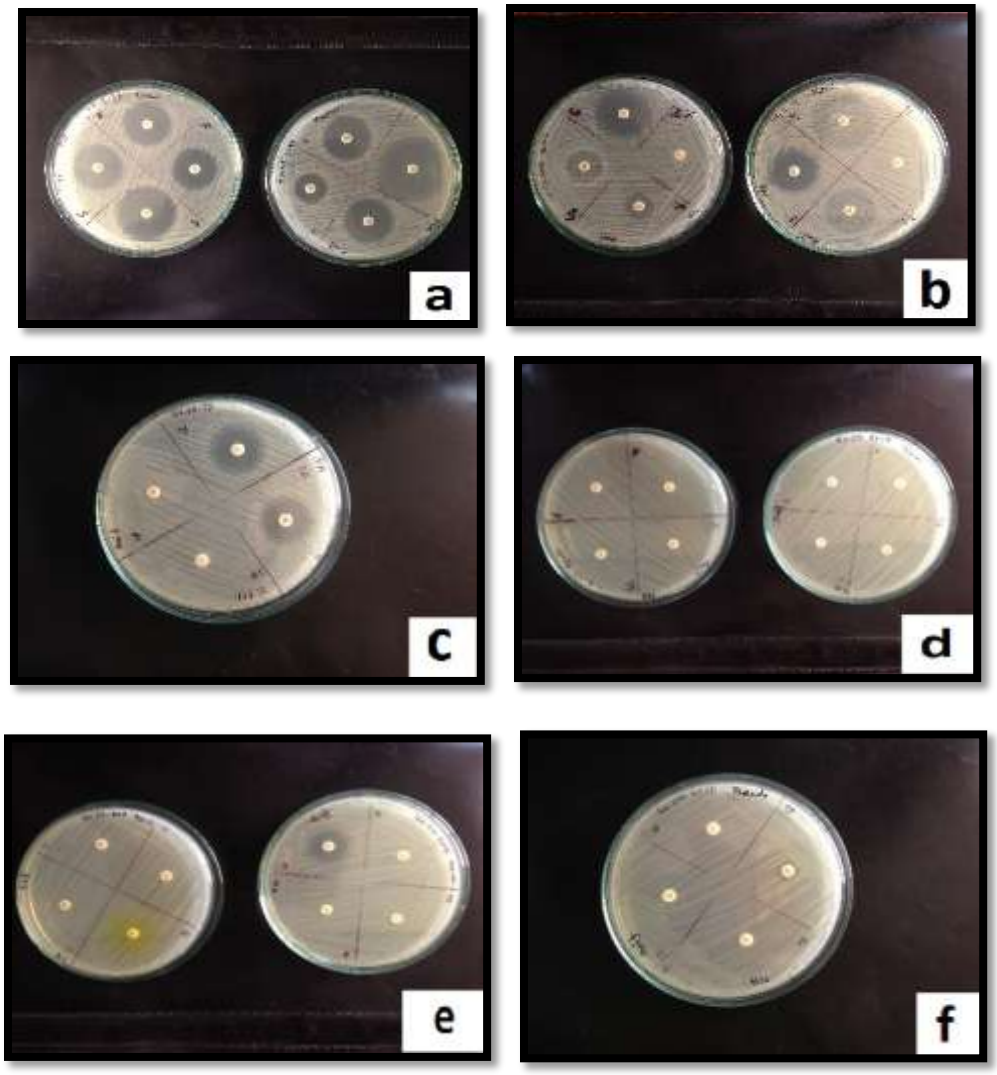


Figure 9: **a.** *Staphylococcus aureus* showing high sensitivity to amoxicillin, cefoxitin, ceftazidime, ceftriaxone, cmipenem, chloramphenicol, Clindamycin, Doxycycline. **b.** *E. coli* showing resistance to amoxicillin, cefoxitin, ceftriaxone and clindamycin, **c.** *E. coli* showing resistance of oxacillin and Penicillin-G

d. *Pseudomonas spp*. showing resistance to amoxicillin, cefoxitin, ceftazidime, ceftriaxone, cephalexin, chloramphenicol, clindamycin and doxycycline. **e.** *Pseudomonas spp.* is only intermediate to Imipenem. **f.** *Pseudomonas spp.* showing resistance to tobramycin, streptomycin, rifampicin and penicillin-G.

Chapter 4

Discussion

4.1 Discussion

Effectiveness of hygienic practice and hygienic status in the production of dairy food products are reflected by its total bacterial count, fungus count and coliform organisms present. These are commonly used methods for evaluation of quality control of food products. A variety of diseases can potentially be transmitted through milk and other milk products. The pathogenic agent source can be cow or humans associated with the production and handling of the foods and it can be transmitted into other milk products (Pelczar, 2007).

In a previous study Bhowmick *et al,* (2006) found that the range of coliform bacteria in borhani (spicy yogurt milk) was 0.5×10^4 - 5.24×10^5 cfu/ml. The current research study also showed similar result. The coliform count obtained from the study was $1.0 \times 10^4 – 8.5 \times 10^6$ cfu/ml. It can be seen that current research result showed more coliform bacteria count which indicated severe contamination in the food products.

Another study was reported by Baraheem *et al,* (2007) where they investigated other dairy products like ice-cream, butter milk, etc. Among the liquid samples, the highest coliform count was seen in butter milk sample which was 1.4×10^4 cfu/ml. Similarly, current study showed the mean coliform count for plain butter milk was 7.5×10^3 cfu/ml which is way higher than the previous study conducted by Baraheem *et al,* (2007).

Malek *et al,* (2015) conducted a research study and they included matta (plain butter milk), sweetened yogurt, lassi and borhani (spicy yogurt milk) as the samples. In their study, all the samples exhibited the presence of bacterial contamination. Among specific pathogens they spotted *Staphylococcus spp*. and *Klebsiella spp*. Similar result was also found in current study. After several biochemical tests, presence of *Staphylococcus spp*. and *Klebsiella spp*. were confirmed in samples of spicy yogurt milk and plain butter milk collected from food markets in Dhaka city. However, in the study of Malek *et al,* (2015) they have found *Vibrio spp.* at a very high rate in the samples which is dissimilar with present study. Present investigation also showed presence of other pathogenic microorganisms such as *Salmonella spp., Pseudomonas spp.* which does not correspond with the study conducted by Malek *et al,* (2015).

Another study was done by Khan *et al,* (2008) on raw milk of balk tank milk before it is processed to other milk products such as butter milk and curd. In their study, they found that the bulk tank milk was highly contaminated by *Escherichia coli* and *Pseudomonas aeruginosa* species. The presence of these pathogenic isolates was also confirmed in the after product where the milk was used. Like the study of Khan *et al,* (2008), in present research study *Escherichia coli* and *Pseudomonas spp.* were found in plain butter milk and spicy yogurt milk. So, it can lead to an assumption that the contamination occurred from the beginning of the food processing.

Ahmed *et al,* (2015) conducted a study on flavoured yogurt and investigated the microbial quality. The samples were prepared and stored. The study was carried out to identify the cause of contamination and the microorganisms responsible. They found that all the samples were highly contaminated with *Staphylococcus aureus.* According to their study, it was suggested that environmental contamination during preparation and handling of the products might be the reason of the contamination. In current research study *Staphylococcus aureus* was found in spicy yogurt milk samples. This result was similar to the previous finding because the contamination might have happened while handling the food. *Staphylococcus aureus* is widely found in respiratory tract, nose and skin. Therefore, food those are exposed or frequently touched could be contaminated by it. This result was also supported by Aly *et al*. (2004) where *Staphylococcus aureus* count got higher due to frequently exposure. In another study by Belickonva *et al,* (2001), *S. aureus* count was reported in yogurt milk and strawberry flavored yogurt milk.

Another study was conducted place by Beukes *et al,* (2000) where they investigated dairy products and fermented milk products. During the study, all the samples were tested to confirm the presence of *Staphylococcus aureus, Salmonella spp.*, *Pseudomonas spp.* and *Listeria monocytogenes.*In there study, the IDF reference method (International Dairy Federation, 1995) was used for detection of *Salmonella* species with XLD (Xylose Lysine Desoxycholate) medium as selective solid media. *Staphylococcus aureus* was detected by using the reference method of IDF (International Dairy Federation, 1990). The present study was also similar to the previous one. Presence of *Staphylococcus aureus* and *Salmonella spp.* was confirmed from the dairy product samples. However, *Pseudomonas* spp. was not found in any sample from the study

conducted by Beukes *et al,* (2000) but in this present study, presence of *Pseudomonas spp.* was confirmed.

Present research reviled the high contamination of almost all the samples with *Bacillus spp.* and *Streptococcus spp*. This finding was similar to that of Umoh (1989). According to the study, the yogurt samples and butter milk samples were contaminated with yeast and bacteria which included *Bacillus subtilis*, *Bacillus cereus*, *Streptococcus pyogenes* and other *Streptococcus spp.* The contamination could take place because of the spore forming species. The spores can remain stable and intact in any harsh environment which could be the reason for the proliferation in milk products. Umoh (1989) also reported that because of improper processing, handling and packaging could be the reason for contamination.

The current study showed that multi-drug resistant microorganisms were also present in the collected samples. *Escherichia coli* showed resistance to amoxicillin, cefoxitin, ceftriaxone, clindamycin, oxacillin, penicillin-G as well as *Salmonella spp.* showed resistance to 9 different antibiotics. *Pseudomonas spp*. was surprisingly found to be the most resistant to antibiotics. It showed resistance to twenty antibiotics and was only intermediate to imipenem. However, *Staphylococcus aureus* was the most sensitive among the isolates. In this current study, about 59% isolates showed sensitivity, 36.7% showed resistance and 4.3% showed intermediate susceptibility against the antibiotics used. Nevertheless, only one *Pseudomonas spp.* showed 100% resistance against all the antibiotics. This study was almost similar to the study done by Bhowmick *et al,* (2006). In that study about 60% were sensitivity, 33.33% were resistant and 6.66% were intermediate. Gentamicin and streptomycin showed highest sensitivity. However, imipenem showed the highest sensitivity in present result. The current study results are also closely linked with a previous study conducted by Malek *et al,* (2015). The research group showed some of the isolates were multi-drug resistant. Results of this study also showed a close link with previous study conducted by Marjan *et al,* (2014) where some of the pathogenic isolates exhibited the MDR phenotype.

4.2 Conclusion

Milk is as essential part of our diet and so are the dairy products. In terms of sensory appeal, South Asian people have adopted the spicy yogurt milk and plain butter milk as popular beverages. Having considered the wide acceptance of these beverages, the significance of maintaining hygiene during their preparation and storage is vital. But this report revealed the poor standards of food hygiene which needs to come in light. The previous study findings and present study showed the presence of a wide range of pathogenic bacteria which are public health significance. The current study clearly indicated poor hygiene and management of the food processing. To overcome this problem and minimize the health risks the government authorized institutions such as BSTI should monitor the local markets and food shops. Not only that, the study also shed light into the emerging threat into antimicrobial resistance. All the government, as well as non-government organizations should take some initiative to aware people about the risks so that people aware themselves and others before they consume those products. This can be one of the many possible ways of reducing foodborne illnesses.

Chapter 5

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Chapter 6

Appendices

Appendix- I

Media compositions

The composition of all media used in the study is given below.

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Mannitol Salt Agar

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Xylose-Lysine-Deoxycholate Agar

Component	Amount (g/L)
Yeast extract	3.00
L-lysine	5.00
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.00
Sodium deoxycholate	2.50
Sodium thiosulfate	6.80
Ferric ammonium	0.80
Phenol red	0.08
Agar	15.00

MacConkey Agar

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatin	17.00
Lactose	10.00
Bile salts	1.50
Crystal violate	0.001
Neutral red	0.03
Agar	15.00

M-FC Agar

Component	Amount (g/L)
Tryptose	10.00
Proteose peptone	5.00
Yeast extract	3.00
Lactose	12.50
Bile salts mixture	1.50
Sodium chloride	5.00
Aniline blue	0.10
Agar	15.00

Physiological Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Starch Agar

Component	Amount (g/ L)
Beef extract	3.0
Soluble starch	10.0
Agar	12.0

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0

Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Methyl red Vogus Prekaure (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Gelatin Broth

Component	Amount (g/L)
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
Final pH	6.8 ± 0.2 at 25°C

Nitrate Reduction Broth

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Blood Agar Base

Component	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 0.2 at 25°C

Eosine Methylene Blue Agar

Component	Amount (g/L)
Peptone	10.00
Dipotassium phosphate	2.00
Lactose	5.00
Sucrose	5.00
Eosin yellow	0.14
Methylene blue	0.065
Agar	13.50

Cetrimide Agar

Component	Amount (g/L)
Pancreatic digest of gelatin	20.000
Magnesium chloride	1.400
Potassium sulphate	10.000
Cetrimide	0.300
Agar	15.000
Final pH	(at 25°C) 7.2±0.2

Mueller Hinton Agar

Component	Amount (g/L)
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH	(at 25°C) 7.3±0.1

Appendix - II

Reagents

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temeperature.

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediamine dihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Nitrate Reagent A (100 ml)

5Nacetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water.In a reagent bottle, 0.6 g of N, N-Dimethyl-α-naphthylamine was added along with 100 ml of acetic acid (5N)and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N)^{a} to form a colourless solution and stored at 4°C.

MacFarlane turbidity standard no. 5

Sulfuric acid	0.18 M
Barium chloride	0.048 M
Distilled water	1000 ml

Appendix - III

Gadgets

List of gadgets that were used during the study

Instrument	Manufacturer
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Spectrophotometer, UV mini - 1240	Shimadzu Corporation, Australia
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
Microscope	A. Krüssoptronic, Germany
UV Transilluminator, Model: MD-20	Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland

Appendix - IV

List of abbreviation

TVC	Total Viable Count
TSC	Total Staphylococci Count
TFC	Total Fecal Count
MSA	Mannitol Salt Agar
NA	Nutrient Agar
EMB	Eosin Methylene Blue Agar
CA	Cetrimide Agar
XLD	Xlylose Lysine Deoxycholate Agar

Mac	MacConkey Agar
MIU	Motility Indole Urea
TSI	Triple Sugar Iron Agar
ml	Milliliter
µl	Microliter
mg	Milligram
gm	Gram
Kg	Kilogram
e.g.	For example
et al.	And others
pH	Negative logarithm of hydrogen ion concentration
CFU	Colony Forming Unit
spp.	Species
%	Percentage
°C	Degree Celsius
BCSIR	Bangladesh Council of Scientific and Industrial research
BSTI	Bangladesh Standards and Testing Institution
Sec	Second
mm	Millimeter
µm	Micrometer